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Original Research Article

Advantages of Eva Green real-time mPCR compared to culture methods for differentiating *C. jejuni / coli* directly from feces

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Abstract

The objective of this study is to develop and optimize a rapid molecular method for diagnosing campylobacteriosis directly from a clinical fecal sample and at the same time determining the most common causing agents– *C. jejuni/coli*. 38 clinical fecal samples of hospitalized patients in the Pediatric Clinic of Infectious Diseases – Sofia were tested, the patients aged between 0 and 7 years and with diarrheal syndrome. The clinical samples were tested using a rapid immunochromatographic test (ICT) (*CerTest*Biotec). All positive samples were tested for confirmation by culturing in a microaerophilic atmosphere at 42 °C and subsequently the isolates were biochemically differentiated. The Eva Green real-time mPCR reaction of a direct fecal sample was conducted using the "IQ5TM Real-Time PCR System" apparatus. Out of 38 clinical fecal samples which were ICT positive, 18 strains were isolated by culture – 17 of *C. jejuni* and 1 of *C. coli*. The Eva Green real-time mPCR reaction also reported 18 positive samples for Campylobacter – 17 out of which were of *C. jejuni* and only one of *C.coli*. All other samples were negative for *Campylobacter* spp. The analytical sensitivity and specificity of the mPCR method were 100%. We developed and optimized the Eva Green real-time mPCR for detection and species differentiation of *C. jejuni/coli* directly from a clinical fecal sample. This analysis ensures the faster and more reliable detection of bacterial cells when compared to the conventional culture methods using biochemical differentiation.

Keywords: *Campylobacter,* diarrhea, mPCR

INTRODUCTION

Enteropathogenic bacteria are the cause of acute infectious diarrhea, which is a global health problem. Every year there are over 1.5 billion cases registered of patients with diarrheal syndrome, *Campylobacter* being the leading cause of bacterial gastroenteritis (3,7). In our country routine tests for *Campylobacter* spp. Are not yet performed on diarrheal patients, and when done, it is by using traditional culture methods, which are slow, laborious and capricious. This leads to a delay in the etiological diagnosis, the absence of antibiotic therapy or, on the contrary, polypragmasy with administration of unnecessary antibiotics. Very rarely the microbiological laboratories in Bulgaria perform species characteristics of the most common strains –*C. jejuni* and *C. coli*, thus making epidemiological studies on campylobacteriosis difficult.

Increasingly used in the clinical practice are the immunochromatographic methods, which are fast, cheap

Table 1. Sequence of the primers used and the size of their amplicons.

Gene	Sequence	Amplicon	Source
16S rDNA	CACGTGCTACAATGGCATAT GGCTTCATGCTCTCGAGTT	108 bp	Botteldoorn et al.
ceuE- F	CAAGTACTGCAATAAAAACTAGCACTACG	72 bp	Jun Kawase et al.
ceuE- R	AGCTATCACCCTCATCACTCATACTAATAG		
cj041-F	GATACCTTAAGTGCAGCCTGTGA	74 bp	Jun Kawase et al.
cj041- R	ACGCCTAAACCTATAGCTCCTTC		

and easy to use (Asseva et al., 2012; Boyanova et al., 2004). In most EU countries and North America the molecular methods are routinely used in the diagnostic process (Center of Disease Control, 2008; Sahin et al., 2015; World Health Organization, 2008). In this context, the aim of our work is to develop and optimize a rapid molecular method to diagnose campylobacteriosis directly from a clinical fecal sample and at the same time determine the causing organism down to the species.

MATERIALS AND METHODS

For the period August 2016 – October 2016 38 clinical fecal samples of hospitalized patients aged between 0 and 7 years and suffering from diarrheal syndrome at the Specialized Hospital for Infectious Diseases – Sofia were tested. The clinical samples were tested using a rapid immunochromatographic test (ICT) (*CerTestBiotec*, Spain) for the Campylobacter antigen. The testing was performed for around 2-4 min. depending on the manufacturer's instructions. All positive samples were tested for confirmation using culturing. The cultures were grown in a microaerophilic atmosphere (5%O_2+10%CO_2+ 85%N_2), generated by a gas pack – "Helico-Campy Pack" in an anaerobic jar (Oxoid USA, Columbia, MD)at 42 – 43°C for 24 – 72 hours. The isolates were biochemically differentiated by a positive reaction of sodium hippuratehydrolysis and/or a positive reaction of indoxyl acetate hydrolysis for *C. jejuni* and a positive reaction of indoxyl acetate hydrolysis and a negative reaction of sodium hippurate hydrolysis for *C. coli*.

DNA extraction

Bacterial DNA from the clinical fecal samples was extracted using a ready-for-use kit QUIampDNA BloodMiniKit (QUIAGEN, Germany) in accordance with the manufacturer's instructions.

Eva Green real-time multiplex PCR

The approved PCR method for the simultaneous detection and differentiation of C. jejuni/coli directly from a fecal sample is based on three pairs of primers, which until now were not included together in a common PCR reaction: primers –AB F2/R2, amplifying a specific region of the cj041 gene for *C. jejuni*, and ceuE F/R, amplifying a specific region of the ceuE gene for *C. Coli*, the primers pair camp F2/R2, amplifying a specific region of the genus gene for *Campylobacter spp*. The primers used and their sequences are provided in Table 1. Two reference strains are also included: 33559ATCCfor*C. coli* and 335560 of *C. jejuni*, used as positive controls and ddH_20 as a negative control.

The Eva Green real-time mPCR analysis is optimized to be conducted in a final reaction volume of 25 µl at the specified primers and conditions 1 x Taq DNA Polymerase buffer; 2,5 x DMSO; 0,2 mMdNTP; 0,03/µl HS Taq DNA Polymerase (Applichem GmbH, Germany); 0,005µ Mcamp - F2/R2; 0,55µ MceuE - F/R ; 0,4 µ MAB – F/R.

The Eva Green real-time mPCR reaction takes place in a "IQ5TM Real-Time PCR System" (BIORAD) under the following conditions: the first step is denaturation for 10 min. at 95 ° C, followed by 29 cycles, each consisting of 15 sec. of denaturation at 90ºC, 20 sec. of 58 °C hybridization and 25 sec. of 60 °C elongation; the final step of elongation – 5 min. at 72 °C. The resulting amplicons measure 108 bp, 72 bp and 74 bp in size, these DNA fragments corresponding to the camp genes for Campylobacter spp., CeuE for*C. coli* and cj0414 for *C. jejuni*.

Determining the analytical sensitivity and specificity of the Eva Green real-time mPCR method. For determining the analytical specificity, a group of commonly isolated gastroenteritis-causing bacteria was selected: *Yersinia enterocolitica; Escherichia coli stx1d; E. coli O104; Shigellaflexneri; Salmonella Typhi; Clostridium difficile; Staphylococcus aureus*toxC. The following strains were used as a positive control: ATCC 33560 –*C. jejuni* and C-14.2 –*C. coli*. A 10x DNA of the cultures of the respective strains was used.

For determining the analytical sensitivity, a series of dilutions of a tenfold bacterial suspension of *C. jejuni* and *C. coli*in saline was used.10 µl of each dilution were used to artificially inoculate feces which were negative for campylobacter. The QUIampDNABloodMiniKit was used for DNA extraction.

Table 2. Positive results of the three laboratory methods used to diagnose campylobacteriosis.

ICT (+)	Culture method (+)	mPCR (+)
37	18 *C. jejuni* - 17 *C. coli* - 1	18 *C. jejuni* - 17 *C. coli* - 1

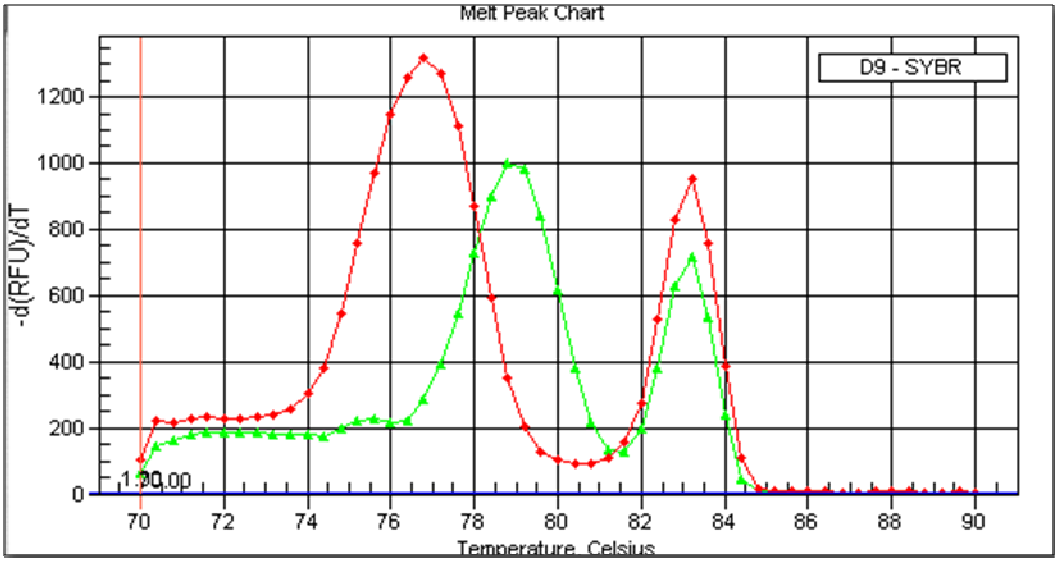

Figure 1. Analysis of the peaks of amplification after aReal-time mPCRatT_a= 58˚ C.
C. jejuni(AB = 78, 8˚ C), *C. coli (ceuE = 76, 8ºC)*

RESULTS AND DISCUSSION

All fecal samples were tested for *C. jejuni/coli* using three separate methods: ICT, culture method and using the Eva Green real-time mPCR. The results obtained are provided in table 2.

A rapid immunochromatographic test (ICT)for the *Campylobacter* antigen has been performed on clinical samples of patients with diarrheal syndrome aged between 0 and 7 years and hospitalized at the Pediatric Clinic of the Specialised Hospital for Infectious Diseases – Sofia, and all positive samples have been verified with a culture and molecular test at the Centre for Infectious and Parasitic Diseases – Sofia.

Out of 38 fecal samples which were ICT positive, 18 strains were isolated in a culture – 17 of *C. jejuni* and 1 of *C. coli.* The strains were identified phenotypically by hydrolysis of sodium hippurate and/or indoxyl acetate. We selected and approved 3 pairs of primers, which up to now had not been included in a general PCR reaction, namely camp F2 –a conservative gene for *Campylobacter* spp., ceuE for *C. coli* and cj0414 for *C. jejuni*. We developed and optimized the Eva Green real-time multiplex PCR for detection and differentiation of species of *C. jejuni/coli* directly from a clinical fecal sample. This analysis provides a rapid and more reliable detection of bacterial cells compared to the conventional culture methods using biochemical differentiation. The amplification curves of the products can be monitored in real time, and that the melting temperatures typical of the target genes are respectively: 78,80 ℃ for *C. jejuni*, 76,80 ℃ for *C. coli* and 82,80 ℃ for *Campylobacter* spp. at the optimal melting temperature of the primers (T_a) of 58 ℃, based on which we optimized the protocol (Figure 1).

Out of 38 clinical samples tested for *C. jejuni/coli,* the PCR analysis reported 18 positive samples for Campylobacter – in 17 of them the PCR products having the typical melting temperatures for *C. jejuni* and only one for *C.coli*. All other samples were negative for *Campylobacter* spp.

Determining the analytical sensitivity of the Eva Green real-time mPCR method

In each PCR reaction for analytical sensitivity we used 7,5µl of concentrated DNA extracted from artificially inoculated feces with serial dilutions of 10 µl of a tenfold bacterial suspension. The analytical sensitivity of the test is approximately 103 CFU per 150 µl of liquid stool. This analytical sensitivity is significantly higher than any

published to date (Boyanova et al., 2004).

The molecular analysis we described has a 100% sensitivity and specificity when comparing the results obtained by it to those of the culture method, which is currently the "gold standard" in the diagnosis of campylobacteriosis. In this study the PCR method is inferior to the ICT only in terms of speed.

CONCLUSION

The fecal samples of patients with acute enterocolitis may contain a variety of pathogenic bacteria. For the purposes of early initiation of etiological treatment, the rapid detection of the bacterial agent causing the particular disease and providing a timely diagnosis are important. We developed the Eva Green real-time mPCR analysis as a rapid, sensitive and specific method for diagnosis of campylobacteriosis directly from a clinical fecal sample and at the same time determining the two most frequently isolated species –*C. jejuni* and *C. coli* for the needs of molecular epidemiology. This method will be very useful in emergency situations such as controlling epidemic situations, in pediatric infectology, as well as in immunosuppressed patients.

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